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## **Supporting Information**

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# Incorporation of Selenomethionine into Proteins via Selenohomocysteine-mediated Ligation

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**General Methods.** All Fmoc-amino acids were from Novabiochem, reagents for peptide synthesis from Applied Biosystems and all other chemicals from Acros, Fluka or Aldrich. LYRAG-SEt was prepared as previously described.<sup>1</sup> Peptides were synthesized on an Applied Biosystems ABI 433A peptide synthesizer using standard HBTU/HOBt/NMP protocols for Fmoc-Chemistry. Amino acid side chains were protected with the following groups: Arg(Pbf), Asn(Trt), Asp(O-*t*Bu), Ser(*t*Bu), Thr(*t*Bu) and Tyr(*t*Bu). Selenohomocysteine was protected as 4-methoxybenzyl (Mob) selenoether (*vide infra*). Preparative RP-HPLC was performed on a Waters HPLC system with 220 nm UV detection using a C18-300 column (Machery Nagel, 250mm × 4.6mm) and a flow rate of 10 ml/min. Solvents: A = CH<sub>3</sub>CN, B = H<sub>2</sub>O/0.1% TFA.

**Selenohomocystine** (adapted from Matthews et al.).<sup>2</sup> To a stirred solution of L-(+)-selenomethionine (3.07 g, 15.7 mmol) in liquid ammonia (150 ml) at –78 °C was added small pieces of metallic sodium (0.84 g, 35.7 mmol) over 20 minutes. After addition was complete the reaction mixture was stirred for 1 h at –78 °C, then solid NH<sub>4</sub>Cl (3.8 g, 71 mmol) was added to neutralize the NaNH<sub>2</sub>. The reaction was allowed to warm to room temperature overnight, during which time the ammonia evaporated. Water (80 ml) was added to dissolve the solids, and N<sub>2</sub> was bubbled through the solution to remove traces of Me<sub>2</sub>Se, which was passed through a washing bottle containing bleach. After 1 h the pH was adjusted to 1 by adding conc. HCl and the solution was concentrated to approximately 40 ml. After filtration, 2N NaOH was added until pH 6. After stirring for 30 min, the solids were filtered off, washed with a small volume of water and dried by lyophilization to give selenohomocystine (2.09 g, 5.7 mmol, 73 %) as a pale yellow solid. Analytical data as published.

**Se-*p*-methoxybenzyl-L-selenohomocysteine hydrochloride.** A suspension of selenohomocystine (1.62 g, 4.5 mmol) in 0.5 N NaOH (4 ml) was cooled in an icebath. A solution

of NaBH<sub>4</sub> (1.44 g, 38 mmol) in H<sub>2</sub>O was carefully added over 15 min. After stirring for 10 min, glacial acetic acid was added to pH~6. 4-Methoxybenzyl chloride (1.2 ml, 8.8 mmol) was added in one aliquot and the reaction was stirred for 30 min at 0 °C. Conc. HCl was added until the pH was 1-2. The white precipitate was collected and recrystallized from hot water to give Se-*p*-methoxybenzyl-L-selenohomocysteine hydrochloride (1.98 g, 5.8 mmol, 65%) as white crystals. m.p. > 200 °C (decomp.). <sup>1</sup>H-NMR (300 MHz, 1 N DCl in D<sub>2</sub>O) δ 2.23 (m, 2H), 2.63 (t, 2H, J = 7.2 Hz), 3.84 (s, 3H), 3.88 (s, 2H), 4.17 (t, 1H, J = 6.5 Hz), 6.98 (d, 2H, J = 8.7 Hz), 7.34 (d, 2H, J = 8.7 Hz); ESI MS: m/z 337.7; calcd for C<sub>12</sub>H<sub>17</sub>ClNO<sub>3</sub>Se [M-H]<sup>+</sup>: 337.7.

**N<sup>α</sup>-tBoc-Se-*p*-methoxybenzyl-L-selenohomocysteine.** To Se-*p*-methoxybenzyl-L-selenohomocysteine (1.97 g, 5.8 mmol) was added 1 N NaOH (11.4 ml), H<sub>2</sub>O (3.5 ml), dioxane (10 ml) and a few drops of DMF to solubilize the starting material. The solution was cooled in an ice bath, Boc<sub>2</sub>O (1.4 g, 6.4 mmol) was added and the solution was stirred for 15 min at 0 °C, followed by 1h at room temperature. Dioxane was evaporated and ethyl acetate (50 ml) was added. The solution was cooled in an ice bath and 1 M KHSO<sub>4</sub> was added until pH 2-3. The ethyl acetate layer was separated, washed with H<sub>2</sub>O (3×20 ml), brine (20 ml) and dried (Na<sub>2</sub>SO<sub>4</sub>). After evaporation of the solvent N<sup>α</sup>-tBoc-Se-*p*-methoxybenzyl-L-selenohomocysteine (1.57 g, 3.6 mmol, 62%) was obtained as a clear oil, which was pure as judged by NMR. [α]<sub>D</sub> = -4° (MeOH, c = 0.67); <sup>1</sup>H-NMR (300 MHz, dmsO-d<sub>6</sub>) δ 1.36 (s, 9H), 1.89 (m, 2H), 2.44 (t, 2H, J=7.5 Hz), 3.70 (s, 3H), 3.73 (s, 2H), 3.97 (m, 1H), 6.83 (d, 2H, J= 8.7 Hz), 7.11 (d, 1H, J= 8.4 Hz, NH), 7.17 (d, 2H, J = 8.4 Hz); <sup>13</sup>C-NMR (75 MHz, dmsO-d<sub>6</sub>) δ 20.38, 26.30, 28.86, 32.50, 54.08, 55.66, 78.73, 114.40, 130.50, 132.10, 156.31, 158.56, 174.49; HRMS (MALDI) calcd for C<sub>17</sub>H<sub>25</sub>N<sub>1</sub>Na<sub>1</sub>O<sub>5</sub>Se<sub>1</sub> (M+Na): 426.0790, found: 426.0785.

**LYRAG-selenohomocystine.** LYRAG-SEt (9.94 mg, 11.7 μmol) and selenohomocystine (2.3 mg, 6.8 μmol) were placed under N<sub>2</sub>, and deoxygenated ligation buffer (6M Gn·Cl/100 mM sodium phosphate, pH 9.2, 5 ml) was added. After addition of thiophenol (150 μL, 3 % v/v) the reaction mixture was shaken for 72 h. The mixture was washed with excess ether (3×) to remove thiols. Last traces of ether were removed by blowing N<sub>2</sub> over the solution for 30 min. Purification by preparative RP-HPLC (5% B for 10 min, then increasing to 35% over 50 min) followed by lyophilization gave LYRAG-selenohomocystine (9.2 mg, 5.4 μmol, 92 %). ESI-MS: m/z 1485.6; calcd. average isotope composition for C<sub>60</sub>H<sub>97</sub>N<sub>18</sub>O<sub>16</sub>Se<sub>2</sub> ([M+H]<sup>+</sup>): 1484.4.

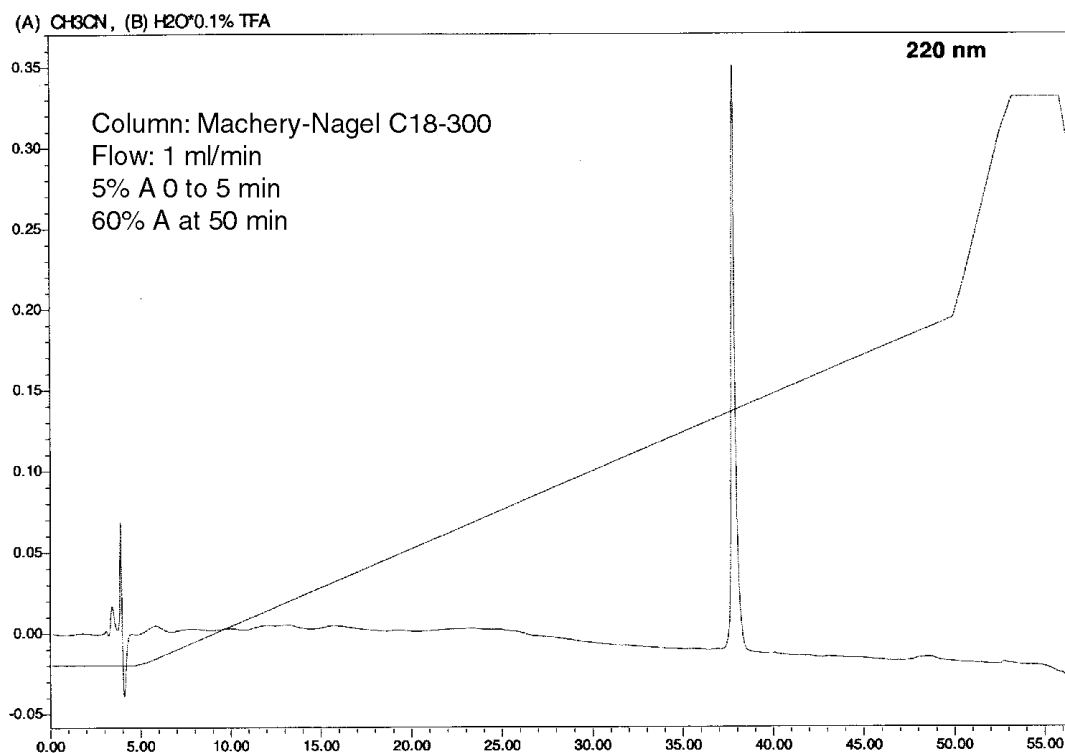
**LYRAG-selenomethionine.** Tris(2-carboxyethyl) phosphine hydrochloride (3.6 mg, 12.4  $\mu\text{mol}$ ) and LYRAG-selenohomocystine (5.3 mg, 6.2  $\mu\text{mol}$ ) were placed under  $\text{N}_2$ . Deoxygenated 200 mM sodium phosphate, pH 6.0 (2.5 mL) was added, followed by a solution of methyl 4-nitrobenzenesulfonate (6.75 mg, 31  $\mu\text{mol}$ ) in deoxygenated acetonitrile (2.5 mL). After stirring for 30 min, a 25% TFA in  $\text{H}_2\text{O}$  (1 mL) was added and the solution was washed three times with ether. Last traces of ether were removed by blowing  $\text{N}_2$  over the solution for 30 min, immediately followed by purification by preparative RP-HPLC (5% B for 10 min, then increasing to 35% over 45 min) and lyophilization to give LYRAG-selenomethionine (4.0 mg, 4.1  $\mu\text{mol}$ , 66 %). ESI-MS:  $m/z$  758.1; calcd. average isotope composition for  $\text{C}_{31}\text{H}_{52}\text{N}_9\text{O}_8\text{Se}_1$  ( $[\text{M}+\text{H}]^+$ ): 757.8.

**bPP(1-16)-SEt (1b).** Peptide **1b** (APLEPEYPGDNATPEQ-SEt) was synthesized on a sulfamylbutyryl AM resin, which was preloaded with Fmoc-Gln(Trt) using published procedures. The *N*-terminal alanine was introduced as an *N*<sup>ε</sup>-Boc protected derivative. For release of the thioester a modification of the procedure by Pessi et al.<sup>3</sup> was used. Resin bound **1b** (193 mg, theoretical loading 0.25 mmol/g) was weighed into a 15 mL polypropylene syringe (containing a 20  $\mu\text{m}$  polyethylene filter) and washed with THF. After swelling the resin in THF, 1 M  $\text{TMSCHN}_2$  (4 mL, hexane/THF 1:1) was added and the mixture was shaken for 3 h. The resin was then washed with THF and a fresh batch of the alkylation solution added, followed by shaking for another 3 h. The resin was washed with THF (4 $\times$ 5 mL) and DMF (4 $\times$ 5 mL) and shaken overnight in a solution containing DMF (4 mL), EtSH (0.7 mL) and a catalytic amount of NaSPh. The whole procedure (alkylation + thioesterification) was repeated, the peptide fractions were combined and treated with a cleavage cocktail (TFA 88.5%, EtSH 4%,  $\text{H}_2\text{O}$  4%, PhOH 2%, thioanisole 1%, triisopropylsilane 0.5%) for 2 h. After removal of the TFA the crude peptide was precipitated and washed with cold ether. Pure **1b** (35.1 mg, 18.6  $\mu\text{mol}$ , 38%) was isolated by preparative RP-HPLC (5% B for 10 min, then increasing to 40% over 50 min). ESI-MS:  $m/z$  1771.8; calcd. average isotope composition for  $\text{C}_{77}\text{H}_{115}\text{N}_{18}\text{O}_{28}\text{S}$  ( $[\text{M}+\text{H}]^+$ ): 1772.9.

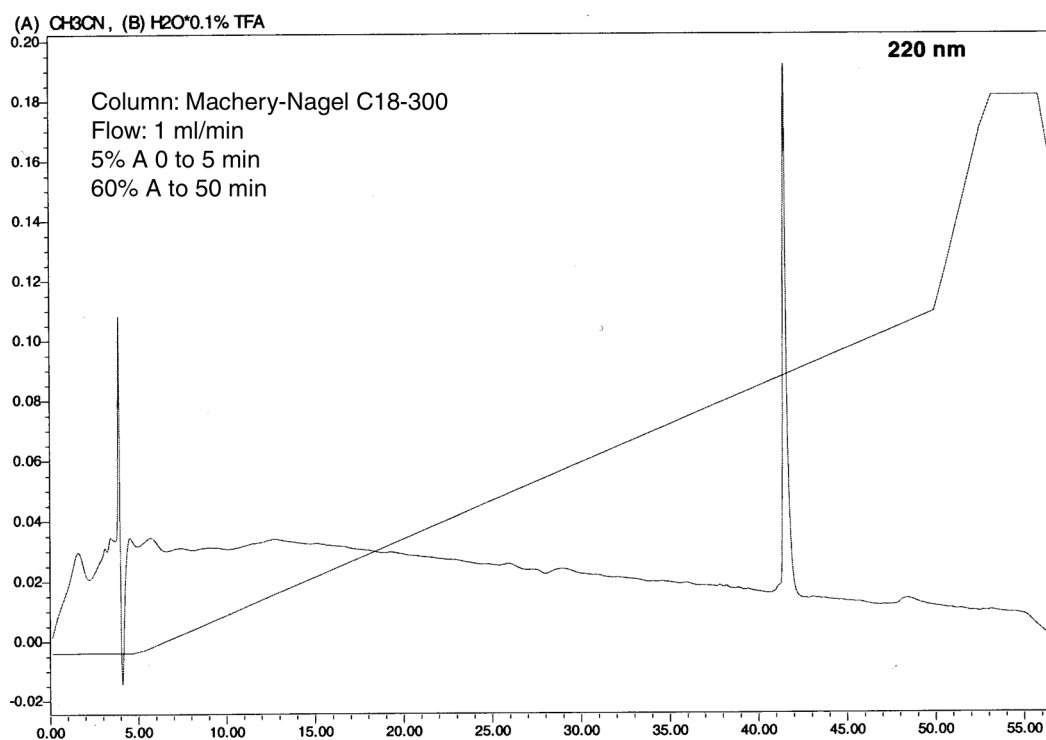
**bPP(17-36) (2b).** Peptide **2b** (selenohomocysteine-AQYAAELRRYINMLTRPRY-NH<sub>2</sub>) was synthesized on a Rink Amide Resin. The *N*-terminal selenohomocysteine was introduced as an *N*<sup>ε</sup>-Boc protected derivative. Part of the resin bound peptide (137 mg, theoretical loading 0.17 mmol/g) was treated with cleavage cocktail (TFA 88.5%, EtSH 4%,  $\text{H}_2\text{O}$  4%, PhOH 2%, thioanisole 1%, triisopropylsilane 0.5%) for 2 h at room temperature. The mixture was concentrated under reduced pressure and a mixture of TFA/*m*-cresol/thioanisole (50:110:75) was added. The solution was placed under  $\text{N}_2$  and cooled in an icebath. TMS-Br (600  $\mu\text{L}$ ) was added and the mixture was shaken at 0 °C for 1 h. All volatile components were removed by

evaporation. The crude peptide was precipitated and washed with cold ether and subsequently purified by preparative RP-HPLC (20% B increasing to 40% over 85 min) to give pure **2b** (16.4 mg, 2.6  $\mu$ mol, 11% overall yield) as the diselenide. The relatively low yield was due to the presence of large amounts of truncated peptides, as a result of difficult stretches in the synthesis. No evidence for methionine oxidation was found. ESI-MS:  $m/z$  5092.8; calcd. average isotope composition for  $C_{220}H_{353}N_{70}O_{56}S_2Se_2$  ( $[M+H]^+$ ): 5096.7.

**Selenohomocysteine-mediated peptide ligation.** **1b** (18.4 mg, 2.93  $\mu$ mol = 5.87  $\mu$ mol monomer) and the diselenide of **2b** (44.0 mg, 4 equivalents) were placed under  $N_2$ . Deoxygenated ligation buffer (100 mM Tris-HCl, 6M GdmCl, pH 8.5, 6ml) and thiophenol (180  $\mu$ L, 3 % v/v) were added, and the mixture was shaken for 4 days. After addition of a second aliquot of thiophenol (180  $\mu$ L), shaking was continued for another 3 days. The mixture was then washed with excess ether (3 $\times$ ) to remove thiols, and the last traces of ether were removed with a stream of  $N_2$ . Purification by preparative RP-HPLC on a C18-300 column (10 ml/min, 5%  $H_2O$ /0.1% TFA in  $CH_3CN$  for 10 min, then increasing to 45%  $H_2O$ /0.1% TFA over 70 min and maintaining for 10 min) afforded the pure ligation product **4b** as a diselenide dimer (9.3 mg, 0.96  $\mu$ mol = 1.93  $\mu$ mol monomer) and a mixed selenosulfide with thiophenol (7.1 mg, 1.53  $\mu$ mol) in a combined yield of 59 %. ESI-MS: diselenide dimer  $m/z$  8516.4, calcd. average isotope composition for  $C_{370}H_{569}N_{106}O_{112}S_2Se_2$  ( $[M+H]^+$ ): 8516.2; selenosulfide  $m/z$  4365.8, calcd. average isotope composition for  $C_{191}H_{290}N_{53}O_{56}S_2Se$  ( $[M+H]^+$ ): 4366.8.

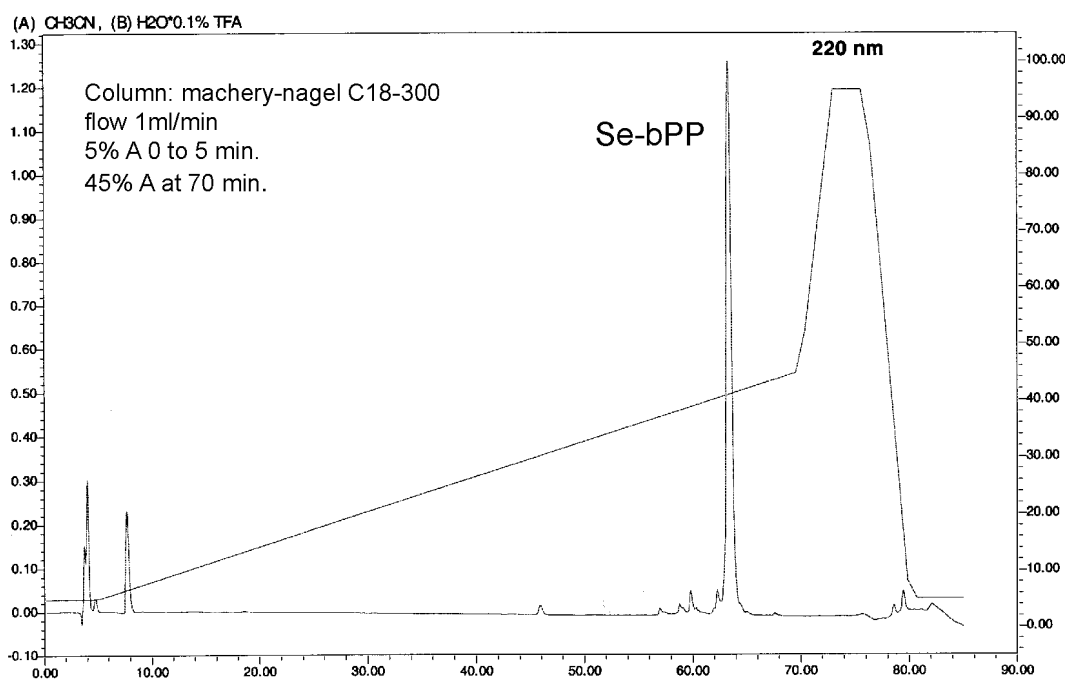


**Figure 1a.** Analytical HPLC-diagram of the purified ligation product diselenide.



**Figure 1b.** Analytical HPLC-diagram of the purified ligation product, the mixed selenosulfide with thiophenol.

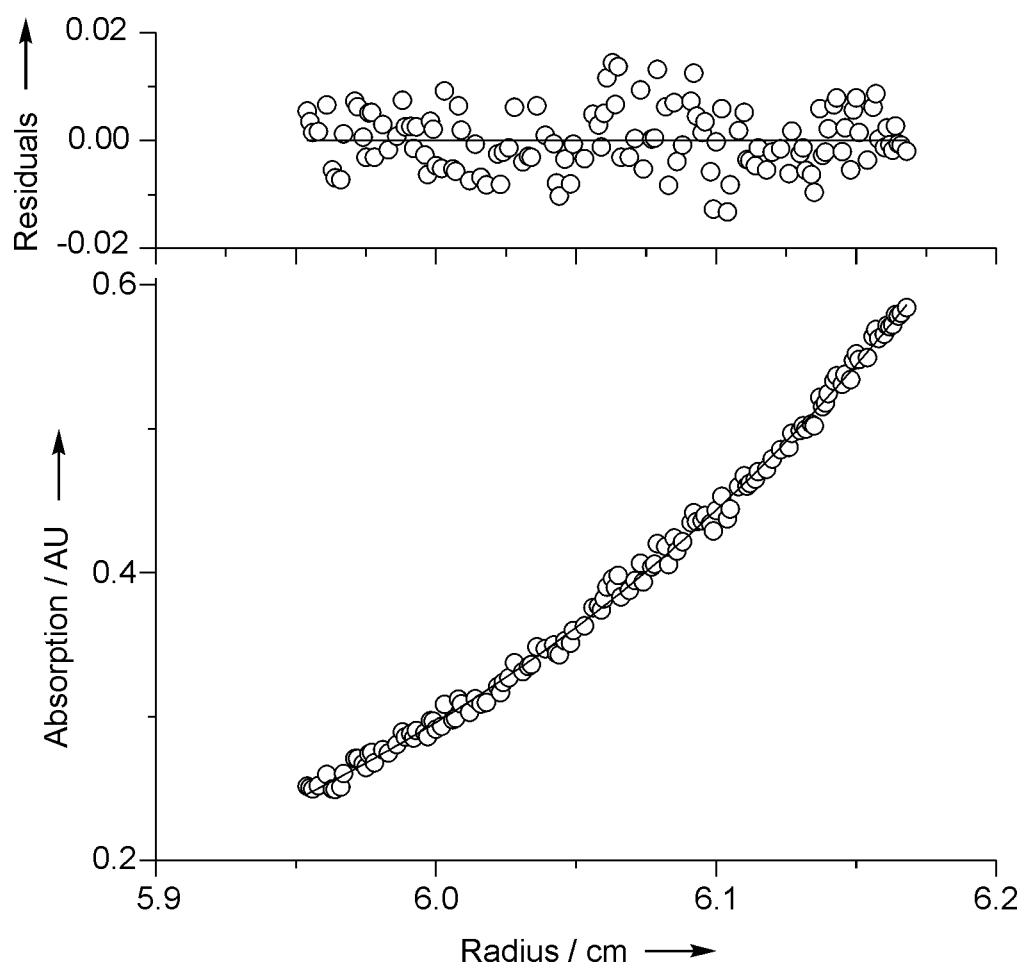
**Methylation of selenohomocysteine.** The diselenide dimer of **4b** (8.7 mg, 0.90  $\mu\text{mol}$  = 1.80  $\mu\text{mol}$  monomer) and tris(2-carboxyethyl) phosphine hydrochloride (1.0 mg, 2 eq based on monomer) were placed under  $\text{N}_2$  and deoxygenated 200 mM sodium phosphate buffer, pH 6.0 (1.8 mL) was added, followed by a solution of methyl 4-nitrobenzenesulfonate (3.8 mg, 10 eq based on monomer) in deoxygenated acetonitrile (1.8 mL). After stirring for 30 min, a 25% solution of TFA in  $\text{H}_2\text{O}$  (1 mL) was added and the solution was washed three times with ether. Last traces of ether were removed by a stream of  $\text{N}_2$  and the crude product purified by preparative RP-HPLC on a C18-300 column (10 mL/min, 5%  $\text{H}_2\text{O}$ /0.1% TFA in  $\text{CH}_3\text{CN}$  for 10 min, then increasing to 45% over 85 min and maintaining for 10 min) to give pure seleno-bPP (**5b**, 6.8 mg, 1.40  $\mu\text{mol}$ , 78 %). ESI-MS:  $m/z$  4272.2; calcd. average isotope composition for  $\text{C}_{186}\text{H}_{288}\text{N}_{53}\text{O}_{56}\text{SSe}$  ( $[\text{M}+\text{H}]^+$ ): 4273.6.



**Figure 2.** Analytical HPLC-diagram of the crude methylation reaction.

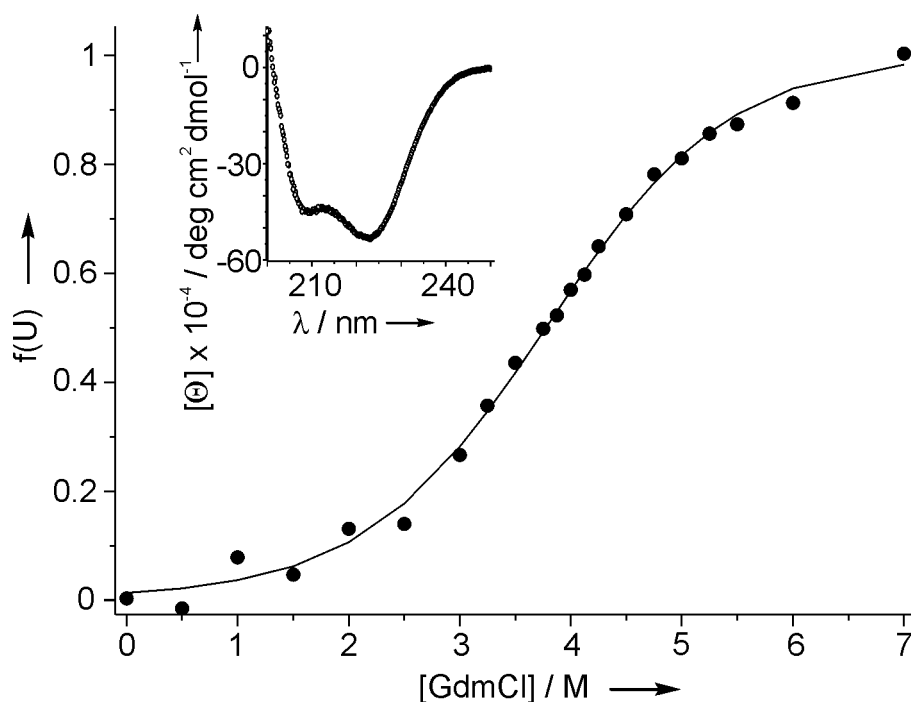


**Sedimentation equilibrium analysis of seleno-bPP.** A 280 mM solution of the protein in 50 mM acetate pH 4.2 was run at 25000, 3000 and 35000 rpm. The data were fitted by a model assuming a single ideal species to give apparent masses of  $8492 \pm 113$ ,  $8398 \pm 81$  and  $8413 \pm 105$ , respectively (Calcd mass of the monomer 4272 g/mol). The apparent partial specific volume used in the studies was  $0.720\text{cm}^3/\text{g}$ , obtained by assuming that the partial contribution of selenomethionine was not significantly different to that of methionine. The effect of C-terminal amidation was ignored. Both partial volume and solvent density were calculated in the normal manner.<sup>[4]</sup>



**Figure 3.** Sedimentation equilibrium analysis of seleno-bPP.

**Chemical Denaturation.** GdmCl-induced denaturation of seleno-bPP (30  $\mu$ M) was carried out in 50 mM acetate buffer, pH 4.2 at 20  $^{\circ}$ C. The fraction of unfolded protein (f(U)) was determined by CD, measuring the ellipticity at 222 nm.



**Figure 4.** GdmCl-induced denaturation of seleno-bPP monitored by CD spectroscopy at 222 nm. Inset: Circular dichroism spectrum of seleno-bPP (30  $\mu$ M).

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